

## THIO-siRNA APTAMERS

## TECHNICAL FIELD OF THE INVENTION

[0001] The present invention relates in general to the field of thioaptamers, and more particularly, to thioaptamers for drug discovery, evaluation and characterization of physiological pathways that silence or interfere with gene expression.

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## BACKGROUND OF THE INVENTION

[0002] This application is a continuation-in-part and claims priority based on U.S. Provisional Application Serial No. 60/105,600, filed October 26, 1998, United States Patent Application, 09/425,798, filed October 25, 1999, now U.S. Patent No. 6,423,493, United States Patent application, 09/425,804, filed October 25, 1999, and United States Patent Application, 10/272,509, filed October 16, 2002. The U.S. Government may own certain rights in this invention pursuant to the terms of the DARPA (9624-107 FP) and NIH (A127744). Without limiting the scope of the invention, its background is described in connection with oligonucleotide agents that interfere with mRNA translation.

[0003] RNA interference (RNAi) is one type of gene silencing in which duplex RNA, either endogenous to cells or delivered exogenous to the cells, interferes with the function of an exogenous or an endogenous gene through a complex form of hybridization to and cleavage of a target mRNA transcript. In the first report of RNAi, in studies on *C. elegans*, it was noted that: (1) interference was observed only for a double-stranded RNA (dsRNA) (not for single-stranded RNA (ssRNA)) sequence within the region of homology of the target gene; (2) that only a few molecules of dsRNA were required per affected cell, arguing against stoichiometric interference with endogenous mRNA and suggesting an amplification component in the interference mechanism; (3) that dsRNA segments corresponding to intron and promoter sequences do not produce interference (which is consistent with a post-transcriptional mechanism of gene silencing); and (4) dsRNA produces a pronounced decrease or elimination of the endogenous mRNA transcript (Fire, et al., 1998).

[0004] It is now known that RNAi is a manifestation of a broader group of post-transcriptional RNA silencing phenomena common to most eukaryotes that can be used to suppress expression of virtually any gene. RNAi, also called "RNA silencing," reflects an elaborate cellular apparatus

that eliminates abundant but defective mRNAs and defends against molecular parasites such as transposons and viruses. Indeed, the main physiological function of RNAi is assumed to be defense against viral infections (Gitlin 2002). Transcription of the silenced gene is unperturbed, but the mRNA transcript for the gene fails to accumulate to its normal cytoplasmic level. Thus, the gene is copied to mRNA in the nucleus, but the mRNA is destroyed, probably in cytoplasm, as soon as it is made.

[0005] Research in gene silencing in plants demonstrated that the silenced plants always contained small RNAs of about 25 nucleotides in length, derived from the sequence of the silenced gene. Such small RNAs are never found in plants that do not display silencing. The small RNAs include both sense and anti-sense fragments of the silenced gene. Similar small RNAs are found in extracts of insect cells pretreated with dsRNA. These “small interfering RNAs” are double-stranded and they are chopped from longer dsRNA by an ATP-dependent ribonuclease called “Dicer.”

[0006] Work in mammalian cell culture using dsRNA of between 38-1662 bp has failed to induce specific RNA interference. Indeed, long double-stranded nucleic acids, such as poly IC, have been known to induce the innate immune response (interferon inducer), whereas shorter double-stranded nucleic acids less than 25 nucleotides (nt) apparently do not induce interferon. However, 21-23 nt dsRNA (siRNAs) having overhanging 3' ends, do mediate sequence-specific mRNA degradation in cultured mammalian cells (Elbashir 2001a, McCaffrey 2002, Caplen 2001). Thus, in humans, siRNAs are 21-23 nt dsRNA generally bearing two-nucleotide 3'overhanging ends. Synthetic siRNAs with the structure of the Dicer products are now routinely used to trigger gene silencing in cultured human cells.

[0007] What is needed are methods and compositions that permit for the rapid detection, isolation and evaluation of siRNA oligonucleotides that have reduced susceptibility to nucleases, that are sequence specific, have an activity that is equal to, or modified from, the activity of a wild-type siRNA or that has one or more activities that are not available for a wild-type RNAi molecule.

#### SUMMARY OF THE INVENTION

[0008] The present invention permits the rapid detection, isolation and evaluation of small RNA oligonucleotides that have reduced susceptibility to nucleases, that are sequence specific, have a gene silencing activity that is equal to, or modified from, the activity of, e.g., a wild-type small,

interfering RNA (siRNA); a micro, interfering RNA (miRNA); a small, temporal RNA (stRNA) or even a short, hairpin RNA (shRNA). The compositions and methods of the present invention include “thio-modified nucleotide aptamers” or “thioaptamers” that specifically bind to a target molecule or portion thereof and mediate gene silencing. The effects of thioaptamer binding may be detected at a variety of levels and using a variety of read-outs as disclosed herein and as known in the growing art of RNA interference. Generally, modulation of the functional attributes of bioactive targets is achieved initially by specific thioaptamer binding followed by interference with translation or degradation of a target. Binding may, for example, interrupt protein-DNA, protein-RNA, RNA-DNA, RNA-RNA and/or DNA-DNA interactions such as those that occur between a DICER complex and RNA in the modification of gene expression.

[0009] The present invention includes an isolated thioaptamer that mediates gene silencing. The thioaptamer may include, e.g., a terminal 3' hydroxyl group and include ribonucleotides or deoxyribonucleotides. The portion of the thioaptamer that is modified may include one or more of the following, rATP( $\alpha$ S), rUTP( $\alpha$ S), rGTP( $\alpha$ S), rCTP( $\alpha$ S), rATP( $\alpha$ S<sub>2</sub>), rUTP( $\alpha$ S<sub>2</sub>), rGTP( $\alpha$ S<sub>2</sub>) or rCTP( $\alpha$ S<sub>2</sub>), alone or in combination. The thioaptamer may be made using a method in which a polymerase, e.g., a DNA, an RNA polymerase or even a reverse transcriptase is used to incorporate the rNTP's with thiophosphate substitutions so that the thioaptamer has the monothioate or dithioate substitutions. Generally, the thioaptamer will be from about 21 to about 25 nucleotides in length, however, modification of the thioaptamer intracellularly may decrease or increase the length of actual active gene silencing thioaptamers. The thioaptamers of the present invention may be, e.g., a double stranded thioaptamer with a perfect complementarity match to a target gene wherein gene silencing occurs by mRNA cleavage; a thioaptamer with an imperfect complementarity match to a target gene wherein gene silencing occurs by repressed translation of mRNA to protein; or a single-stranded thioaptamer with perfect complementarity match to a target gene wherein gene silencing occurs by mRNA cleavage. The thioaptamer may be a portion of a RNA-induced silencing complex (RISC) complex and/or produced by a DICER complex.

[0010] In one embodiment, the thioaptamer may be, e.g., a short interfering RNA (siRNA); a micro, interfering RNA (miRNA); a small, temporal RNA (stRNA); or a short, hairpin RNA (shRNA). In some cases, the thioaptamer provided to a cell or cellular extract may be a thioaptamer precursor, e.g., a long dsRNA or an about 70 nucleotide stem-loop RNA (shRNA). Mature thioaptamers will generally be a double stranded thioaptamer of about 21 to about 25 nucleotides long or a single-stranded thioaptamer that is about 15 to about 22 nucleotides long or

even up to about 28 nucleotides long. In one embodiment, gene silencing may be by degradation of an mRNA transcript that is cleaved in the presence of the thioaptamer before it can express a protein. Alternatively, gene silencing may be accomplished by the regulation of translation when the thioaptamer binds an mRNA transcript at or about its 3'UTR.

5 [0011] The present invention also include a method of producing a mature thioaptamer of from about 21 to about 23 nucleotides in length that includes the steps of, combining a double-stranded precursor thioaptamer with a soluble extract that mediates gene silencing, thereby producing a precursor-extract mixture; and maintaining the precursor-extract mixture under conditions in which the double-stranded thioaptamer is processed to the mature thioaptamer of from about 21  
10 to about 23 nucleotides in length. The method may also include isolating the thioaptamer of from about 21 to about 23 nucleotides from the precursor-extract mixture. The method may also include the step of determining the sequence of the mature thioaptamer and the location of one or more thio-modifications to the mature thioaptamer. Upon isolation, selection or improvement of selective binding the method may further include the steps of, determining the sequence of the  
15 mature thioaptamer and the location of one or more thio-modifications to the mature thioaptamer; and chemically synthesizing the mature thioaptamer, e.g., a mature thioaptamer of about 21 to about 23 nucleotides that is produced by the method disclosed herein.

[0012] Another method of the present invention is mediating gene silencing of a target gene in a cell or organism by introducing a thioaptamer of from about 21 to about 23 nucleotides in length  
20 into the cell or organism and maintaining the cell or organism under conditions in which gene silencing occurs, thereby mediating expression of the target gene in the cell or organism. In one example, the thioaptamer may be optimized for RNase H degradation and thereby cause gene silencing. Examples of target genes include, endogenous and exogenous genes (e.g., viral or cellular genes), transgenes and the like. The compositions and methods of the present invention  
25 may be used to make a knockdown cell or organism to, e.g., mimic a disease. Target cells may include cells in any stage of development, e.g., stem cells. Using the thioaptamers disclosed herein, the function of a gene may be examined in a cell or organism by introducing a thioaptamer of from about 21 to about 23 nucleotides that targets an mRNA of the gene for gene  
30 silencing into the cell or organism, thereby producing a test cell or test organism; maintaining the test cell or test organism under conditions under which gene silencing of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is silenced and observing the phenotype of the test cell or test organism against an appropriate control cell or control organism to provide information about the function of the gene.

[0013] The present invention also includes a method of assessing whether a gene product is a suitable target for drug discovery by introducing an RNA thioaptamer that mediates gene silencing of from about 21 to about 25 nucleotides into a cell or organism under conditions in which gene silencing of an mRNA for the target gene results in decreased expression of the gene; and determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery. In one embodiment, the thioaptamer may be part of a pharmaceutical composition, e.g., a thioaptamer of about 21 to about 25 nucleotides that mediates thioaptamer gene silencing and an appropriate carrier.

[0014] The thioaptamers of the present invention may also be used as part of a method of identifying target sites within an mRNA that are efficiently targeted for gene silencing by combining an RNA thioaptamer corresponding to a sequence of a labeled mRNA to be degraded under conditions in which labeled mRNA is degraded. Next, the sites in the mRNA that are efficiently cleaved are identified. The RNA thioaptamer may be part of a thioaptamer library, e.g., a pool of thioaptamers from a thioaptamer library or even a library of libraries. In an alternative method, target sites may be identified within an mRNA that are efficiently targeted for gene silencing by combining an RNA thioaptamer corresponding to a sequence of a labeled mRNA under conditions in which labeled mRNA is not degraded and the protein level is reduced.

[0015] The present invention also includes a combinatorial thioaptamer library that includes two or more unique thioaptamers that include a combination of backbone modifications and sequence that mediates gene silencing of an mRNA to which it corresponds. The thioaptamers may be attached covalently to one or more beads, e.g., polystyrene/polydivinyl benzene copolymer. The thioaptamers may include one or more phosphorothioate linkages, one or more phosphorodithioate linkages and/or one or more methylphosphonate linkages. The thioaptamer may include, e.g., a viral sequence, a genomic sequence and/or an expressed sequence. The thioaptamers may also include a detectable agent, e.g., a colorimetric, a fluorescent, a radioactive and/or an enzymatic agent. The thioaptamers disclosed herein may also include a strand complementary to the thioaptamer. The library of thioaptamers may be, e.g., created by a split and pool combinatorial synthesis chemistry.

[0016] One example of a library is a one-bead, one-thioaptamer combinatorial library that includes, two or more beads, wherein attached to each bead is a unique thioaptamer comprising a single unique sequence, wherein each unique thioaptamer includes a unique mix of modified and

unmodified nucleotides and wherein the thioaptamer mediates gene silencing of an mRNA to which it corresponds. Alternatively, the one-bead, one-thioaptamer combinatorial library may be two or more beads, wherein attached to each bead is a unique thioaptamer comprising an imperfect complementarity match to a target gene to form a thioaptamer-bead, wherein each  
5 unique thioaptamer-bead comprises a unique mix of modified and unmodified nucleotides and wherein the thioaptamer mediates gene silencing of an mRNA to which it has imperfect complementarity. In yet another example, the combinatorial library is a bead library of thioaptamer libraries, wherein each bead comprises a thioaptamer library of imperfect complementarity to a target sequence for gene silencing.

10 [0017] Using the RNA thioaptamers disclosed herein it is possible to reduce the expression of a gene in a cell by selecting a thioaptamer that mediates gene silencing of the gene to which it corresponds and introducing the thioaptamer into the cell, wherein the thioaptamer mediates RNA interference of a targeted sequence. Sequences that may be targeted by the RNA thioaptamers of the present invention include, e.g., gene markers, splice acceptors, splice donors, IRES,  
15 recombinase sites, promoters, ori sequences, cloning sites, and intervening sequence. Target cells include non-mammalian, plant, yeast, bacterial, mammalian, human and even stem cells. The thioaptamer may be an antisense molecule and may even be a ribozyme.

[0018] In one use, the RNA thioaptamers may be used to attenuate expression of a target gene in cultured cells, by introducing an RNA thioaptamer into the cells in an amount sufficient to  
20 attenuate expression of the target gene, wherein the RNA thioaptamer includes a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene and mediates attenuation of protein expression for a gene to which it corresponds. The method may further include the step of activating a gene silencing activity in the cell.

[0019] In one example, RNAi may be used as a potential alternative to transgenic mice, where  
25 the knock-out effect could be turned on and off. Potential limitations in using dsRNA to knock-out a gene function may include: (1) a sequence shared between closely related genes might interfere with several members of the gene family; (2) a low level of expression might resist RNAi for some or all genes; and/or (3) a small number of cells might escape RNAi so that one does not get complete loss of function as one would get with a knockout mouse (Fire, et al.,  
30 1998).

[0020] The present invention may be used to control, study, evaluate or even diagnose a biological pathway using the thioaptamers of the present invention by using the thioaptamer in a method that uses some of the steps below, depending on the nature of the host cell. For example, mammals use steps 2-4, below. In contrast, plants and worms use steps 1-4 in which dsRNA is amplified by RdRPs.

Step 1 – dsRNA is amplified by RNA-dependent RNA polymerases (RdRPs);

Step 2 – dsRNA is chopped up by Dicer to 21-23 nt siRNAs;

Step 3 – the siRNA is incorporated into an RNA-induced silencing complex (RISC) containing an endonuclease, with the siRNA then guiding the endonuclease to the site of its complementary sequence on the mRNA, and the RISC proceeds to cut up the mRNA at that site, destroying the mRNA; and

Step 4 - the siRNA produced by Dicer also acts as a primer in amplifying dsRNA, which is then acted upon by Dicer, producing more siRNA (Step 2). RISC activity is generally believed to be restricted to the cytoplasm.

[0021] Control of gene expression with nucleic acids such as short antisense oligonucleotides (ssDNA targeting homologous complementary mRNA) is a powerful tool for investigating protein function inside cells (Koller, 2000) and may provide a major new class of therapeutics (Jansen and Zangemeister-Wittke, 2002; Opalinska and Gewirtz, 2002; Braasch and Corey, 2002). It has been pointed out that dsRNAs represent a potential addition to therapeutic nucleic acid control of gene expression (Zamore, 2002). Steps 1 and 2 in the above mechanism can be bypassed by transfection of chemically synthesized 21-23 nt dsRNAs, called small interfering RNA or siRNAs. Knowing only the DNA sequence of a gene, one could design sequence-specific siRNA to inhibit expression of that gene. By turning off the mRNA of that gene, one could study the function of that gene. Thus, RNAi has potential both as a therapeutic and as a tool for the study of physiological pathways.

[0022] In vitro studies in human cell culture have demonstrated siRNA inhibition of retroviral infection utilizing delivery of exogenous synthetic siRNA against HIV challenge (Novina, 2002; Jacque, 2002; Lee, 2002), RSV challenge (Hu, 2002) and HCV challenge (Yokota, et al., 2003) and transfection with a plasmid expressing siRNA targeting poliovirus (Gitlin, 2002) and hepatitis C virus (Yokota, et al., 2003). It has also been demonstrated, in mouse models, that siRNAs can function in vivo, inhibiting gene expression of both endogenous genes (Wianny 2000, Xia 2002, McCaffrey 2002, Song 2003) and exogenous genes (McCaffrey 2003). The latter report demonstrated siRNA inhibition of hepatitis B virus in mice. It has also been shown

in mammalian cell models that siRNA can be used to target disease-causing mutant alleles (e.g., single-nucleotide polymorphisms (SNPs)) of genes suggesting therapeutic application to SNP-linked diseases (Miller, et al., 2003).

5 [0023] It has been demonstrated that siRNA-mediated gene silencing is sufficiently specific and reliable to allow large-scale screening of gene function and drug target validation via gene expression profiling following siRNA delivery (Semizarov, et al., 2003). In another study, microarray gene expression studies in siRNA gene silencing did not indicate detectable off-target gene silencing, as had previously been reported in *C. elegans* work (Chi, et al., 2003). In another study using Dharmacon-supplied siRNAs, however, off-target silencing was observed (Jackson, 10 et al., 2003), which may have been due to excess siRNA in the cell, indicating that siRNA levels must be optimized (RNAi Roundup article on GenomeWeb internet site).

[0024] A study comparing siRNA to antisense oligonucleotides for gene knockdown indicated that in terms of dose-response, siRNA had an  $IC_{50}$  that was 100-fold lower than that of antisense oligonucleotides, and that as is the case for antisense, siRNA efficacy differed at different target 15 sites on the mRNA target (Miyagishi, et al., 2003). It is difficult to predict a priori the most effective target site for a siRNA design, however, it was observed that siRNAs generated in vitro by recombinant human Dicer typically have high RNAi activity (Kawasaki, et al., 2003), offering an experimental optimization path.

#### BRIEF DESCRIPTION OF THE DRAWINGS

20 [0025] For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures in which:

Figure 1 is a gel that shows the titration of RNA aptamers and a VEE Capsid protein;

25 Figures 2A, 2B and 2C are stem-loop structures for three aptamers including a variant of the 16\_1 aptamer, the 7-7 aptamer and a third related aptamer, respectively;

Figure 3 is a gel of the titration of the RNA aptamer 16\_1 with the VEE Capsid Protein;

Figure 4 is a graph that demonstrates the specificity of the 16\_1 RNA aptamer;



Figure 5 summarizes the selection modification cycle used to prepare RNA thioaptamers that combine sequence specificity and thio-modification to the aptamers;

Figure 6A, 6B, 6C and 6D are stem-loop structures for three engineered RNA aptamers derived from 16\_1;

- 5 Figure 7 is a graph that shows the effect of thio-modification of the aptamer on siRNA gene silencing in HeLa cells;

Figure 8 is a graph that shows the effect of thio-modification of the aptamer on siRNA gene silencing in HeLa cells;

Figure 9 is a graph that shows the effect of thioaptamers on siRNA gene silencing in HeLa cells;

- 10 Figure 10 is another graph that shows the effect of thioaptamers on siRNA gene silencing in HeLa cells; and

Figure 11 is a graph that shows the silencing by thioaptamers in HeLa cells.

#### DETAILED DESCRIPTION OF THE INVENTION

- 15 [0026] While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

- 20 [0027] To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

- 25 [0028] As used herein, “synthesizing” of a random combinatorial library refers to chemical methods known in the art of generating a desired sequence of nucleotides including where the desired sequence is random. Typically in the art, such sequences are produced in automated

DNA synthesizers programmed to the desired sequence. Such programming can include combinations of defined sequences and random nucleotides.

[0029] "Random combinatorial oligonucleotide library" means a large number of oligonucleotides of different sequence where the insertion of a given base at given place in the sequence is random. "PCR primer nucleotide sequence" refers to a defined sequence of nucleotides forming an oligonucleotide which is used to anneal to a homologous or closely related sequence in order form the double strand required to initiate elongation using a polymerase enzyme. "Amplifying" means duplicating a sequence one or more times. Relative to a library, amplifying refers to en masse duplication of at least a majority of individual members of the library.

[0030] As used herein, "thiophosphate" or "phosphorothioate" are used interchangeably to refer to analogues of DNA or RNA having sulphur in place of one or more of the non bridging oxygens bound to the phosphorus. Monothiophosphates or phosphoromonothioates [ $\alpha$ S] have only one sulfur and are thus chiral around the phosphorus center. Dithiophosphates are substituted at both oxygens and are thus achiral. Phosphoromonothioate nucleotides are commercially available or can be synthesized by several different methods known in the art. Chemistry for synthesis of the phosphorodithioates has been developed by one of the present inventors as set forth in U. S. Patent #5,218,088 (issued to Gorenstein, D.G. and Farschtschi, N., June 8, 1993 for a Process for Preparing Dithiophosphate Oligonucleotide Analogs via Nucleoside Thiophosphoramidite Intermediates), relevant portions incorporated herein by reference.

[0031] As used herein, the terms "thio-modified aptamer" and "thioaptamer" are used interchangeably to describe oligonucleotides (ODNs) (or libraries of thioaptamers) in which one or more of the four constituent nucleotide bases of an oligonucleotide are analogues or esters of nucleotides that normally form the DNA or RNA backbones and wherein such modification confers increased nuclease resistance; and the DNA or RNA may be single or double stranded. For example, the modified nucleotide thioaptamer can include one or more monophosphorothioate or phosphordithioate linkages selected by incorporation of modified backbone phosphates through polymerases from wherein the group: dATP( $\alpha$ S), dTTP( $\alpha$ S), dCTP( $\alpha$ S), dGTP( $\alpha$ S), rUTP ( $\alpha$ S), rATP( $\alpha$ S), rCTP( $\alpha$ S), rGTP( $\alpha$ S), dATP( $\alpha$ S<sub>2</sub>), dTTP( $\alpha$ S<sub>2</sub>), dCTP( $\alpha$ S<sub>2</sub>), dGTP( $\alpha$ S<sub>2</sub>), rATP( $\alpha$ S<sub>2</sub>), rCTP( $\alpha$ S<sub>2</sub>), rGTP( $\alpha$ S<sub>2</sub>) and rUTP( $\alpha$ S<sub>2</sub>) or modifications or mixtures thereof. Phosphoromonothioate or phosphorodithioate linkages may also be incorporated by chemical synthesis or by DNA or RNA synthesis by a polymerase, e.g., a DNA

or an RNA polymerase or even a reverse transcriptase, or even thermostable or other mutant versions thereof. In another example, no more than three adjacent phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In yet another example, at least a portion of non-adjacent dA, dC, dG, dU or dT phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In another example of a thioaptamer, all of the non-adjacent dA, dC, dG, or dT phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups; all of the non-adjacent dA, dC, dG, and dT phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups; or substantially all non-adjacent phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In still another embodiment of the present invention, no more than three adjacent phosphate sites of the modified nucleotide aptamer are replaced with phosphorodithioate groups. The thioaptamers may be obtained by adding bases enzymatically using a mix of four nucleotides, wherein one or more of the nucleotides are a mix of unmodified and thiophosphate-modified nucleotides, to form a partially thiophosphate-modified thioaptamer library. In another example of "thioaptamers" these are made by adding bases to an oligonucleotide wherein a portion of the phosphate groups are thiophosphate-modified nucleotides, and where no more than three of the four different nucleotides are substituted on the 5'-phosphate positions by 5'-thiophosphates in each synthesized oligonucleotide are thiophosphate-modified nucleotides.

[0032] Thiophosphate nucleotides are an example of modified nucleotides. "Phosphodiester oligonucleotide" means a chemically normal (unmodified) RNA or DNA oligonucleotide. Amplifying "enzymatically" refers to duplication of the oligonucleotide using a nucleotide polymerase enzyme such as DNA or RNA polymerase. Where amplification employs repetitive cycles of duplication such as using the "polymerase chain reaction", the polymerase may be, e.g., a heat stable polymerase, e.g., of *Thermus aquaticus* or other such polymerases, whether heat stable or not.

[0033] "Contacting" in the context of target selection means incubating a oligonucleotide library with target molecules. "Target molecule" means any molecule to which specific aptamer selection is desired. "Essentially homologous" means containing at least either the identified sequence or the identified sequence with one nucleotide substitution. "Isolating" in the context of target selection means separation of oligonucleotide/target complexes, preferably DNA/protein complexes, under conditions in which weak binding oligonucleotides are eliminated.

[0034] By “split synthesis” it is meant that each unique member of the combinatorial library is attached to a separate support bead on a two (or more) column DNA synthesizer, a different thiophosphoramidite or phosphoramidite is first added onto both identical supports (at the appropriate sequence position) on each column. After the normal cycle of oxidation (or sulfurization) and blocking (which introduces the phosphate, monothiophosphate or dithiophosphate linkage at this position), the support beads are removed from the columns, mixed together and the mixture reintroduced into both columns. Synthesis may proceed with further iterations of mixing or with distinct nucleotide addition.

[0035] Aptamers may be defined as nucleic acid molecules that have been selected from random or unmodified oligonucleotides (“ODN”) libraries by their ability to bind to specific targets or “ligands.” In one embodiment, an iterative process of in vitro selection may be used to enrich the library for species with high affinity to the target. The iterative process involves repetitive cycles of incubation of the library with a desired target, separation of free oligonucleotides from those bound to the target and amplification of the bound ODN subset using the polymerase chain reaction (“PCR”). The penultimate result is a sub-population of sequences having high affinity for the target. The sub-population may then be subcloned to sample and preserve the selected DNA sequences. These “lead compounds” are studied in further detail to elucidate the mechanism of interaction with the target.

[0036] Thioaptamers and other nucleic acid analogs (e.g. peptide nucleic acids (PNAs), methylphosphonates, etc.) are emerging as important agents in therapeutics, drug discovery and diagnostics. Three key attributes define the unique ability of (thio)aptamers to perform their essential functions: (1) they target specific proteins in physiological pathways; (2) their sequence and structure is not intuitively obvious from canonical biologics and oftentimes can only be deduced by combinatorial selection against their targets; and (3) they bind their targets with higher affinities than do naturally occurring nucleic acid substrates. Importantly, the backbone modifications of thioaptamers and their nucleic acid backbone analogs enable aptamers to be introduced directly into living systems with in vivo lifetimes many times greater than unmodified nucleic acids, due to their inherent nuclease resistance of the modified aptamers. The inherent nuclease resistance is extraordinarily important for their efficacy in use.

[0037] The term “gene silencing” as defined herein is used to describe the phenomenon of reduced or repressed translation of mRNA into a protein. Examples of thioaptamer mediated “gene silencing” include short ssDNA, ssRNA or dsRNA, that may vary from 15 to 70 nt long

(for precursors) that repress protein expression by specific or non-specific degradation of mRNA and/or binding to the mRNA in a location, time and manner that inhibits the cellular translational complex from translating the mRNA into protein. Degradation may occur, e.g., by non-specific antisense DNA/RNA duplex formation and resulting RNase H-type RNA degradation or sequence specific DICER/RISC mediated mRNA degradation. The term "RNA interference" (RNAi) is defined herein as gene silencing by cleavage of perfectly complementary mRNA, which in mammals is mediated by 21-23 nt small, interfering RNAs (siRNAs) which are double-stranded, and which are produced by Dicer cleavage of long ds RNA, with the resulting siRNA incorporated into an RNA-induced silencing complex (RISC). As used herein, the term gene silencing also applies to miRNA repression of translation, in which the miRNA complementarity is imperfect but the thioaptamers of the present invention are able to repress (lower or eliminate) gene translation.

[0038] Table 1 summarizes the types of gene silencing that may be achieved using the thioaptamers of the present invention. For example, gene silencing may be by cleavage of perfectly complementary mRNA mediated not only by siRNAs, but also by 21-22 nt, single-stranded miRNAs. The thioaptamer may be designed and selected, e.g., based on the target strandedness of the message or the thioaptamer and may be double- or single-stranded, which the skilled artisan will recognize as the distinguishing characteristics between a miRNA and a siRNA.

[0039] Table. 1 Summary of Thioaptamer Gene Silencing Described Herein

Trigger	Strand	Length	Precursor	Complementarity to target Required	Silencing mechanism
siRNA	ds	21-23 nt (mammals) 21, 25 nt (plants)	long dsRNA	perfect	mRNA cleavage
miRNA/ stRNA	ss	21-22 nt (eukaryotes)	70 nt stem-loop RNA (shRNA)	imperfect	repress translation of mRNA to protein
miRNA	ss	21-22 nt	70 nt stem-loop RNA (shRNA)	perfect	mRNA cleavage

siRNA=small, interfering RNA  
miRNA=micro, interfering RNA  
stRNA=small, temporal RNA  
shRNA=short, hairpin RNA

[0040] The thioaptamers of the present invention may operate by transcriptional silencing through which mRNA is not produced by the gene target and by post-transcriptional silencing. Two examples of post-transcriptional gene silencing, include: (1) an mRNA that is produced by

transcription but is then cleaved/degraded by an siRNA or miRNA before it can express protein; and (2) an mRNA that is produced by transcription and is not cleaved/degraded, but its translation into protein is repressed/regulated by binding of a miRNA to, e.g., its 3'-UTR. Gene silencing by repression of the translation of mRNA targets to protein by the thioaptamers described herein may be mediated by single-stranded microRNAs (miRNAs) which are 21-22 nt long and are homologous but not perfectly complementary to the target mRNA, bind to the 3'-UTRs of the target mRNA, and are produced by Dicer cleavage of circa 70 nt long ("short") "hairpin" RNA precursors. As such, the thioaptamers and the libraries of thioaptamers described herein (e.g., the library of libraries) may include thioaptamer shRNA precursors that are then "processed" into the mature "gene silencing" thioaptamer by Dicer. Such imperfectly complementary miRNAs are also called "small, temporal RNA (stRNA)." miRNA repression of translation has been identified in plants, worms, flies and mammals. Precursor "gene silencing" thioaptamers may be single- or double-stranded.

[0041] A "target gene" as defined herein may be, e.g., a gene derived from the cell, a transgene (e.g., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen that is capable of infecting an organism from which the target cell is derived. Depending on the particular target gene and the dose of thioaptamer delivered, this process may provide partial or complete loss of function for the target gene. In some cases, gene silencing of a target gene may be a reduction or loss of gene expression in at least 99% of targeted cells.

[0042] Generally, gene silencing may be shown by the inhibition of gene expression such that the level of protein and/or mRNA product from a target gene in a cell is absent or reduced about 5, 10, 20, 30, 50, 75 80, 90 or even about 100% (i.e., an observable decrease within the limits of detection of the assay selected to measure gene silencing). Specificity of the thioaptamer refers to the ability of the thioaptamer to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition may be confirmed by examination of phenotypic changes (i.e., outward properties of the cell or organism) or by genotypic or biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For thioaptamer-mediated inhibition in a cell line or whole organism, gene expression may be assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Reporter genes may include, e.g., acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ),

beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Furthermore, the detection of gene silencing may even be by using multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracycline.

[0043] Depending on the assay used to measure gene silencing using the thioaptamers of the present invention, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than about 5%, 10%, 20%, 25%, 33%, 50%, 60%, 75%, 80%, 90%, 95% or about 99% as compared to a target cell that has not been not treated according to the methods of the present invention. The thioaptamers disclosed herein may permit the use of lower doses of injected material and longer times after administration of, e.g., dsRNA thioaptamers resulting in the inhibition of a smaller fraction of cells (e.g., at least about 10%, 20%, 50%, 75%, 90%, or about 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of silencing that depends on the level of accumulation of target mRNA and/or translation of target protein. For example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

[0044] The thioaptamers disclosed herein may be delivered as a double-stranded RNA thioaptamer, as a single self-complementary RNA thioaptamer strand (single-stranded RNA thioaptamer with a tertiary structure, e.g., hair-pin loops) or two complementary RNA thioaptamer strands. RNA thioaptamer duplex formation may be initiated either inside or outside the cell. The thioaptamer may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded thioaptamers may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for gene silencing.

[0045] Thioaptamers having a nucleotide sequence identical to a portion of the target gene will most often be used for gene silencing, however, nucleotide sequences may be varied by insertions, deletions, and single point mutations relative to the target gene sequence. Thus,

sequence identity may be optimized by sequence comparison and alignment algorithms known in the art that calculate the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group (GCG)), ClustalW, etc. The thioaptamers may have a sequence identity greater than 90% with a target sequence, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing at medium to high stringency, as will be known to those of skill in the art (See e.g., Maniatis, et al.) with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C or 70° C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least about 25, 50, 100, 200, 300 or 400 bases for the precursors. As such, 100% sequence identity between the thioaptamer and the target gene is not required to practice the present invention, which allows for tolerate of sequence variations that might be expected due to genetic mutations, polymorphisms, or evolutionary convergence, drift, shift and divergence.

[0046] A cell with the target gene may be derived from or contained in any organism or particle. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Microbes may be, e.g., those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies. Particles may include viruses and the like.

[0047] The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, cloned, immortalized or transformed and the like. The cell may be a stem cell or a differentiated cell and may be derived from a wild-type, a genetic mutant, a genotypic variant, a transgenic, a knock-out, a knock-in and the like. Cell types that are differentiated include, e.g., adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, macrophages, granulocytes, e.g., neutrophils, eosinophils and basophils, mast cells, lymphocytes, e.g., B-cells and T-cells, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

[0048] The thioaptamer may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism,



introduced orally, or may be introduced by bathing an organism in a solution containing the thioaptamer. For example, the thioaptamer may be sprayed onto a plant or a plant may be genetically engineered to express the thioaptamer in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing the thioaptamer may include, e.g., injection directly into the cell or extracellular injection into the organism. Other methods for delivering the thioaptamer include, e.g., bombardment by particles covered by the thioaptamer, soaking the cell or organism in a solution of the thioaptamer or electroporation of cell membranes in the presence of the thioaptamer. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the thioaptamer may be introduced along with components that perform one or more of the following activities: enhance thioaptamer uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

[0049] The thioaptamer may also be used for the treatment or prevention of disease. For example, dsRNA thioaptamers may be introduced into a cancerous cell or tumor and thereby inhibit expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology. Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

[0050] The thioaptamers may also target a gene for immunosuppression of a host. Alternatively, the thioaptamers may be targeted at target genes for replication of a pathogen, transmission of the pathogen, or maintenance of the pathogenic infection. The gene silencing thioaptamer is introduced in cells in vitro or ex vivo and then subsequently placed into an animal to effect therapy, or directly introduced by in vivo administration.

[0051] Research studies comparing siRNA to chemically optimized antisense technology, have indicated that fewer RNA duplexes have to be screened in order to identify active siRNAs, that siRNAs might be more potent inhibitors of gene expression than antisense (Miyagishi, et al., 2003), and that siRNAs were less toxic to cells (Braasch, et al., 2003). Phosphorothioate (PS) modified antisense oligonucleotides are the “gold standard” for antisense therapy, conferring nuclease resistance to these ss DNA oligonucleotides and increasing binding to serum proteins which increases bioavailability (Geary, et al., 2001). A next generation of phosphorothioate anti-

sense oligonucleotides may be based on the introduction of locked nucleic acid bases (LNA) into the molecule to enhance binding affinity (Braasch and Corey, 2002).

[0052] Researchers in the field have studied the effects of similar chemical modification of siRNA for use in RNAi to potentially enhance serum and cellular stability. Partial substitution of dsRNA with either phosphorothioate linkages or 2'-deoxy-2'-fluorouridine nucleotides, on one or both strands has been shown to continue to support RNAi (Parrish, 2001). Whereas extensive 2'-O methyl modification did not support RNAi (Elbashir, 2001b), limited modification did support RNAi (Amarzguioui, 2003). The fluorouridine modification may have an added advantage in preparation of the large amounts of material required by a therapeutic application, in that elimination of the 2'-hydroxyl group simplifies synthesis, deprotection and purification protocols. It has also been observed that whereas minimal substitution with phosphorothioate linkages is tolerated, extensive phosphorothioate substitution is toxic to cells (Prydz, 2003). However, in a separate study with PS modification at 4-21 nucleotides of a 21 nt siRNA, cell toxicity was not observed (Braasch, et al., 2003). The discrepancy between the two reports was ascribed by Braasch as possibly being due to the presence of toxic impurities in the PS-siRNA samples used in the Prydz work. Importantly, there is no method to predict the optimal location of the thiophosphate modification to optimize siRNA activity and serum and cellular stability.

[0053] Research on the effect of a single mutation in a siRNA strand on RNAi activity has indicated that while a mutation on the antisense strand reduces activity, a mutation on the sense strand has no effect. This indicates that the two strands of a siRNA molecule have different functions in RNA interference. Chemical modification of the 3' end of the sense strand exclusively is possible without incurring loss of RNAi activity. However chemical modification at the 3' end of the antisense strand abolished activity, indicating that it is the 3' end of the antisense strand that is recognized by the RISC (Hamada, et al., 2003). The following is a summary of the research on the stability and efficacy of PS-siRNA in mammalian cell culture (Braasch, et al., 2003).

[0054] Stability. Surprisingly, whereas ssRNA is degraded rapidly by serum nucleases, dsRNA was reasonably stable in serum. Since siRNA must remain intact in order to interact with the RISC complex, the dsRNA nuclease resistance presumably facilitates endogenous RNAi. PS-ssRNA substituted with 12 or 21 (complete) PS linkages was degraded in serum. Finally, PS-modified dsRNA was stable in serum, although its stability was not significantly higher than that of unmodified dsRNA.

[0055] Efficacy. Modification with either PS, fluorouridine or LNAs continues to support RNAi and introduction of PS linkages into dsRNA reduced  $T_m$  (78 C to 58-73 C), whereas fluorouridine substitution did not affect  $T_m$  and LNA substitution increased  $T_m$  significantly (78° C to 93° C). Braasch hypothesized that siRNA thermal stability might be important since lower  $T_m$  could cause dissociation and since ssRNA is degraded by nucleases, could reduce RNAi. The  $T_m$  for all cases of substitution, however, is significantly higher than physiological temperature and such hypothesized dissociation may be negligible. Reduction of the melting temperature of the dsRNA, while still keeping  $T_m$  well above physiological temperature, could enhance siRNA activity if unwinding of the duplex is a limiting step in the enzymatic reaction sequence of RNAi. Since dithiophosphate linkages reduce the melting temperature of the siRNA/thioaptamer more than monothiophosphates, the dithiophosphates could be optimal for chemical modification of siRNA. Furthermore, although greater than 80% of cells were successfully transfected with both unmodified and PS-modified dsRNA, the nuclear uptake of PS-modified dsRNA was significantly higher than that of unmodified dsRNA, consistent with an earlier report that PS-DNA/lipid complexes exhibited enhanced nuclear localization (Marcusson, 1998). Finally, PS-dsRNA with 4, 8, 12, 16 and 21 PS linkages inhibited gene expression at low levels (sub-50 nM) whereas unmodified dsRNA activity dropped significantly below 50 nM.

[0056] Although in the study described above, PS-dsRNA did not significantly increase serum stability of the already stable dsRNA, it was hypothesized by the authors that the PS linkages may improve the pharmacokinetics of siRNA (Braasch, et al., 2003), since modification with as few as 13 PS substitutions improves the pharmacokinetics of antisense oligonucleotides by increasing their binding to serum proteins (Geary 2001). It was thus proposed that a few LNA modifications, avoiding the central region of the siRNA, in order to increase thermal stability, should be combined with PS-linkages to improve pharmacokinetics as a strategy for design of a chemically modified siRNA.

[0057] Whereas the aforementioned study indicated that PS-siRNA did not enhance nuclease resistance, at least one developer of siRNAs has argued that without chemical modifications RNAi will not be an option for therapeutics. The same developer has claimed that the ribose sugar makes the siRNA molecule unstable, and thus they have replaced it with a “ribose prosthetic,” e.g., xylofuranosyl modifications into polynucleotides (Matulic-Adamic, et al., 1996). In vitro studies of hepatitis B virus (HBV), by Sirna Therapeutics, e.g., found that standard siRNA was more active than “prosthetic” siRNA at day three post-administration, but the “prosthetic” siRNA was much more active at day 21 post-administration (comments in RNAi

Roundup article on GenomeWeb internet site). Sequitur Inc., developer of a “Stealth RNAi,” has found that standard siRNA molecules transfected into cells undergo degradation in the cytosol within hours, although the triggered RNAi activity persists for days. The so-called “Stealth” RNAi is said to be significantly more stable in the cytosol, allowing the monitoring of siRNA uptake. The inventors of the subject invention believe that these studies indicate a need for increasing the nuclease resistance and in vivo stability of siRNA, as this could enhance dose persistence in a therapeutic setting.

[0058] While others have used these so-called “prosthetic” modifications, the present inventors have reasoned that the enhanced nuclear localization of PS-dsRNA should also increase therapeutic efficacy and the higher RNAi activity at dsRNA levels below 50 nM may also be significant in vivo. Using the present invention, a variation in the number of LNA substitutions and the location of LNA substitutions on a 21 nt siRNA resulted in significant variation in inhibition of gene expression, hypothesized to be due to variation in the ability of the modified LNA-siRNA to be recognized by the proteins comprising the RISC complex (Braasch, et al., 2003). Variation in the number of PS linkages and their position on a 21 nt siRNA targeting human Tissue Factor resulted in significant variation in persistence of gene silencing after 5 days post-transfection with siRNA, whereas PS-siRNA and wildtype-siRNA exhibited similar activity before day 5 (Amarzguioui, 2003).

[0059] Extensive work on thioaptamer recognition of proteins by the inventors of the subject invention, suggests that combinatorial library selection of PS-siRNA, in which the nucleotide sequence is constant, but the number and position of the PS links are combinatorially varied, or in which both backbone and sequence are varied, should yield optimized siRNA for specific applications, and thus for specific RISC complex recognition. Combinatorial library selection allows the identification of thiophosphate patterns that enhance the cellular nuclease stability of the thioaptamer, enhance binding to RISC proteins and catalytic activity in the RISC-siRNA nuclease cleavage of the mRNA transcript. Recognition of the siRNA molecule by the proteins included in the RISC complex is required in order that the separation of the siRNA strands and subsequent recognition of the mRNA target by an siRNA strand can proceed. Current means of optimization of siRNA depend on algorithms based on sets of selection rules, focusing on siRNA sequence in terms of optimum sites on the target gene and avoidance of sites common to a family of proteins and to sites known to activate interferon response – examples are Dharmacon’s 34 rule algorithm ([www.dharmacon.com](http://www.dharmacon.com)) and/or Tushl’s set of selection rules.

[0060] The present invention uses a novel methodology of combinatorial library selection of aptamers in order to select small (30 nt random sequence region), partially thioated RNA aptamers targeting the Venezuelan Equine Encephalitis (VEE) virus. VEE is a likely agent of biological warfare and/or terrorism (BWT) for several reasons:

- 5           (1)    VEE virus is known to have been highly developed as a BWT agent in both the United States and in the former Soviet Union.
- (2)    VEE virus is readily isolated from natural sources.
- (3)    VEE virus replicates to high titer in a variety of cell cultures.
- (4)    VEE virus is highly stable when lyophilized.
- 10          (5)    VEE virus is highly infectious by aerosol; over 150 instances of laboratory aerosol infection have been documented.
- (6)    VEE virus produces a highly debilitating and sometimes fatal disease, with permanent neurological sequelae in many cases.
- 15          (7)    If introduced into a location with susceptible equines and mosquitoes, VEE virus can produce a widespread epidemic.
- (8)    No licensed VEE virus vaccine exists. Current experimental vaccines have poor efficacy and a high rate of adverse reactions.
- (9)    No effective antivirals have been developed against VEE virus.

[0061] Because VEE virus may be used for biological terrorism, the present invention includes  
20 new strategies for antiviral development, focusing on powerful combinatorial methods that allow for rapid selection and identification of lead compounds for cell culture and animal challenge studies. The present invention includes compositions and methods for the rapid isolation, identification, purification, characterization and development of gene silencing thioaptamers, thiophosphate-backbone modified oligonucleotide agents (RNA- and DNA-based  
25 oligonucleotides (ODNs)) to a wide range of proteins and mRNA, including viral proteins that are essential for virion assembly and/or the mRNA transcripts which translate to viral proteins.

[0062] The VEE virus capsid protein is an attractive target protein because it interacts with other capsid protein molecules in nucleocapsid formation, and also interacts with the cytoplasmic tail of the E2 envelope glycoprotein to initiate virion budding. Thus, the initial studies focused on  
30 targeting the capsid protein using the new combinatorial selection technology. The in vitro combinatorial selection methodology has selected ssRNA thioaptamers towards the nucleocapsid protein of VEE virus (all monothiophosphates at the 5'-dA positions). Combinatorial selection of

RNA aptamers targeting VEE virus was implemented with completion being assessed based on convergence of sequence of RNA aptamers with an affinity of 1-2 nM. Table 2 shows the aligned sequences of 23 high affinity thioRNA aptamers (random sequence region is 30 nt), which share considerable sequence identity.

5 [0063] TABLE 2 is a ClustalW Multiple Sequence Alignment of RNA Aptamers

7-2	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
13-A10	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
16-2	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
16-4	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
10 16-8	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
16-16	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
16-7	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
16-5	-CUGCCUACG-CCAUGCCCAGAACCCUCACGC-- (SEQ ID NO.: 1)
7-7	GGCCCUUGCGCCCACACGCAAACACCGCCC---- (SEQ ID NO.: 2)
15 7-11	GGCCCUUGCGCCCACACGCAAACACCGCCC---- (SEQ ID NO.: 2)
16-1	GGCCCUUGCGCCCACACGCAAACACCGCCC---- (SEQ ID NO.: 2)
7-12	-CGCCAACCGACCGUCCCGACCGUCCGCCUC--- (SEQ ID NO.: 3)
16-9	-CGCCAACCGACCGUCCCGACUGUCCGCCUC--- (SEQ ID NO.: 3)
16-12	--UGCCCAGG-CCGCGGCCAUACUACUACGCC- (SEQ ID NO.: 4)
20 13-A9	GCUCAGAUCCCCCGCCCCGCUAUCCGCAC---- (SEQ ID NO.: 5)
13-A11	---UCCUGUGCCCGGACCCUGUCCCCUGCUG--- (SEQ ID NO.: 6)
16-6	--UGCCAA-GUCGGCUUCCAUCCACCACCCGAG- (SEQ ID NO.: 7)
16-15	----CCGACGGAAUUCCCGCUAGUUCCCCUGACC (SEQ ID NO.: 8)
7-14	----CCGACGAC--UGAUUAUUCCCCUGCCCCCA (SEQ ID NO.: 9)
25 16-14	---UCACACCACACGCUUCAUCCCCUGCAC---- (SEQ ID NO.: 10)
7-6	-CACCUCACAACUUCGCACCUCAACCGUCUC--- (SEQ ID NO.: 11)
7-9	----CCCUACGACUUGCUUGCCAGCGGACGCCA (SEQ ID NO.: 12)
7-10	AUGGCUUACAAGCCGCAGCUCUAUGUGGAC---- (SEQ ID NO.: 13)

30 [0064] Although statistically significant homology cannot be established between any of the selected aptamer sequences and the VEE virus genome, significance would not be expected unless the identity is circa 100% for these short sequences. More importantly, cis-acting RNA sequences important for VEE viral replication generally are defined by secondary structure rather than by primary sequences, so homology would not necessarily be expected.

35 [0065] Initial testing of aptamer No. 7-7 did not reveal any evidence of antiviral activity (data not shown), however, a positive control for liposome fusing and RNA entry was not available. Synthesis of a fluorescein-labeled aptamer for evaluating RNA uptake and localization in cells before doing more challenge studies may be used in conjunction with the aptamer to optimize delivery of the aptamer. Once delivery is optimized, the antiviral activity in Vero and BHK cells  
40 may be detected, and the studies in a mouse model of antiviral activity finalized.

[0066] EXAMPLE 1 – Combinatorial selection and characterization of phosphorothioate RNA aptamers against VEE capsid protein. Combinatorial selection of aptamers was employed to isolate RNA aptamers targeting VEE (Venezuelan Equine Encephalitis) virus capsid protein. VEE is a potential bioterrorism agent, and its capsid protein, which plays a major role in viral replication, is a drug target. The combinatorial selection procedure was designed to modify the backbone of RNA aptamers with phosphorothioate linkages. This chemically modified phosphorothioate RNA (PSRNA or thioaptamer) is expected to improve the efficiency and stability of a RNA aptamer as a potential drug. One of the highest affinity thioRNA aptamers from the first generation selection was aptamer 16\_1:

10 5'-GGGAGCUCAGAAUAAACGCUCAAGGCCCUUGCGCCCACACGCAAACACCGCCCU  
UCGACAUGAGGCCCGGAUCCGGC-3' (30 nt random region is underlined)(SEQ ID NO.: 14).

[0067] To develop a second generation aptamer, it was necessary to perform structural mapping (i.e., footprinting) to elucidate the VEE capsid binding site on the aptamer. Figure 1 is a footprinting gel that shows the binding of 16\_1 to the VEE Capsid protein. The chemical footprinting of aptamer 16\_1 was conducted as follows: 10nM of biotinylated aptamer 16\_1 was incubated with variable concentration of VEE capsid protein: Protein concentration of each lane was 0 (lane 4), 1 nM (lane 5), 19 nM (lane 6), 100 nM (lane 7), 1 mM (lane 8) and 10 mM (lane 9). After 2 hours incubation, iodine and ethanol mixture was added to the binding mixture to cleave unprotected phosphorothiolated phosphate bonds in the RNA aptamer. Lane 1 is a protein size marker. Lane 2 is aptamer 16\_1 only. Lane 3 is aptamer 16\_13.

[0068] According to the footprinting result there is no isolated region on the aptamer that binds to VEE capsid protein. To determine the structural region on the thioRNA aptamer that is essential and sufficient for binding, three engineered RNAs were made (Figures 2A, 2B, 2C) from the aptamer 16\_1 and tested their binding capability. Each RNA migrated to show multiple bands in the gel.

KLG\_5\_46:

5'-GGGAGCUCAGAAUAAACGCUCAAGGCCCUUGCGCCCACACGCAAGC-3' (SEQ ID NO.: 15)

KLG 3\_45:

30 5'-GGCCCUUGCGCCCACACGCAAACACCGCCCGCCCGGAUCCGGCC-3' (SEQ ID NO.: 16)

KLG\_M\_45:

5'-GGUUGCGCCACACGCAAACACCGCCCUUCGACAUGAGGCCCGGC-3' (SEQ ID NO.: 17)

[0069] As shown in the gels in Figure 4, only the upper bands of each RNA bound to VEE capsid protein. The binding assay of three engineered RNAs was as follows: 0.5 nM of biotinylated RNA was incubated with variable concentrations of VEE capsid protein. Protein concentration of each lane was: 0 nM (lane 3), 4 nM (lane 4), 8 nM (lane 5), 16 nM (lane 6), 32 nM (lane 7), 64 nM (lane 8), 128 nM (lane 9) and 256 nM (lane 10). After 2.5 hours incubation, the binding mixture was loaded onto the gel. Lane 1 is a protein size marker. Lane 2 is the same as Lane 3 but with 25% formamide added to partially denature the RNA. Binding of the RNA was measured based on the decrease of the upper bands as protein concentration was incremented. From this analysis, KLG\_3\_45 was determined to be the tightest binding aptamer. Based on this result, second generation RNA aptamers may be selected based on modifications of KLG\_3\_45.

[0070] The first generation selection procedure was studied from a system point of view, to characterize the degree of selection achieved by the combinatorial selection procedure. Comparison of the apparent binding constants of phosphate and phosphorothioate forms of the initial library and of the combinatorially selected aptamer 16\_1 to the VEE capsid protein indicated that: (1) selection did not significantly enhance the affinity of unmodified RNA to VEE capsid protein. This can be explained from the fact that VEE capsid protein binds nucleic acids promiscuously; (2) the position of the phosphorothioate modification is a key determinant in selection. Different enhancement of affinity to VEE capsid protein due to the phosphorothioate modification between the initial library and selected aptamer indicate the position of phosphorothioate in the selected aptamer played a role in the selection of the aptamer. For example, the PS-aptamer selected from the PS-library had 5.1-fold higher affinity than the unmodified aptamer selected from the unmodified library, and the PS-aptamer selected had 9.7 times higher affinity than the library whereas the unmodified aptamer had 7.5 times the affinity of its library. These results allow the generation of a model for selection and modification (Figure 5).

[0071] Three further variants of aptamer 16\_1 were developed and the stem-loop structures determined shown in Figures 6A, 6B, 6C and 6D:



Figure 6A:

5'-GGGAGCUCAGAAUAAACGCUCAAGGCCCUUGCGCCCACACGCAAACACCGCCC  
UUCGACAUGAGGCCCCGGAUCCGGCUU-3' (SEQ ID NO.: 18)

Figure 6B:

5 5'-GGGAGCUCAGAAUAAACGCUCAACUGCCUACGCCAUGCCCAGAACCCUCACGG  
UUCGACAUGAGGCCCCGGAUCCGGCUC-3' (SEQ ID NO.: 19)

Figures 6C:

5'-GGGAGCUCAGAAUAAACGCUCAACUGCCUACGCCAUGCCCAGAACCCUCACGC  
UUCGACAUGAGGCCCCGGAUCCGGCUU-3' (SEQ ID NO.: 20)

10 Figure 6D:

5'-GGGAGCUCAGAAUAAACGCUCAUAGCCCAUCCUGC  
UUCGACAUGAGGCCCCGGAUCCGGCUU-3' (SEQ ID NO.: 21)

[0072] The underlined portions show the variance from the 16<sub>1</sub> aptamer, which also contain two new residues at the 3'-end.

15 [0073] Additional thioRNA aptamers targeting the VEE virus capsid protein will be tested for antiviral activity in cell cultures and in animal models. Additional VEE virus targets may also be studied, using the combinatorial selection/thioation methodology. These studies are described below and are illustrative.

20 [0074] The E2 envelope glycoprotein as aptamer target. This protein resides on the tip of the virion spikes, while E1 lies parallel to the envelope (Lescar et al., 2001; Pletnev et al., 2001). E2 is the site of the major antigenic determinants including most neutralizing epitopes, and is likely to interact with cellular receptors like the high affinity laminin receptor (Griffin, 2001). Therefore, E2 represents the best target for disruption of virion binding and entry into cells. To target E2 with thioRNA aptamers, it is possible to: (1) express the extracellular portion of the E2  
25 protein using E. coli in a maltose binding protein fusion form, purified with an amylose column, or using the baculovirus system to preserve glycosylation; and/or (2) isolate E2 from purified VEE virus virions using weak (non-denaturing) detergent treatment followed by affinity column purification or isoelectric focusing column purification. If necessary, digestive removal of the transmembrane and cytoplasmic portions may be used.

30 [0075] The aptamer selection strategy will be essentially the same that the inventors have used for targeting the VEE virus capsid protein. This in vitro combinatorial selection technology is described in detail in a study of selection of aptamers targeting proteins such as NF-kB (King et al., 2002) and in co-pending applications: 07/430,733; 09/425,798; 09/425,804; 10/120,815;

10/214,417 and 10/272,509, relevant portions, sequences and/or thio-modification(s) incorporated herein by reference.

5 [0076] Cis acting RNA sequences in the VEE virus genome. The three cis-acting sequences that are highly conserved among alphaviruses and are believed to interact with VEE virus nonstructural proteins and cellular proteins for viral replication may be targeted by the thioaptamers of the present invention (Schlesinger and Schlesinger, 2001; Strauss and Strauss, 1994). For example, a combinatorial library of RNA thioaptamers may be produced that target those highly conserved regions of this or any other virus for identification and selection.

10 [0077] The 5' end of the VEE virus genome contains two highly stable stem loop structures that are conserved in their secondary structure. These may also be targeted using the thioaptamers of the present invention. Mutagenesis studies to ablate the stem-loops yet preserve the amino acid sequence in the nsP1 protein render the virus noninfectious, confirming the importance of these secondary structures (I. Frolov, S. Weaver, unpublished).

15 [0078] The 26S subgenomic promoter. This sequence is also strongly conserved among togaviruses (including rubella) and presumably interacts with the polymerase for initiation of transcription. The 3' untranslated genome region is highly conserved among alphaviruses and interacts with cellular proteins including the La antigen. These three RNA elements will be used to make direct decoys with high affinity and stability conferred by limited thiolation. The inventors will also introduce both monothiophosphate and dithiophosphate backbone  
20 substitutions in various random positions in the loop region of the RNA elements to enhance the aptamer affinities. These thioaptamers will be tested in a high throughput screening of inhibition of virus replication.

[0079] Combinatorial libraries of aptamers may also be attached to small polystyrene beads (one aptamer sequence per bead) to select for binding to whole VEE virus virions. Purified TC-83  
25 virus will be mixed with bead libraries and, following washing, flow cytometry in combination with anti-E2 monoclonal antibodies will be used to sort beads with high affinity virus binding. The selected pool of beads will be used to amplify a new, enriched library for subsequent rounds of selection. Finally, the selected aptamers will be tested for in vitro and in vivo antiviral activity.

[0080] Testing of aptamers for antiviral activity. Initial testing may be done in cell culture using  
30 TC-83 attenuated VE virus. For aptamers targeted against the E2 protein or whole virus, a mix of a range of aptamer concentrations may be used along with varying amounts of a virus inoculum

may be used and compared with controls, e.g., a scrambled sequence, negative control aptamers, and infect Vero cells with virus at a Multiplicity of Infection (MOI) of 0.1. Following triplicate infections, one step growth curves may be compared with negative controls for detecting significant suppression of virus replication. Antiviral activity may be confirmed with repeat assays and cell specificity can be determined using other cells such as BHK, 293, HeLa, etc. For the thioaptamers designed to directly mimic conserved cis-acting sequences, delivery may be via lipofectin or other cationic liposomes for introduction into the cell cytoplasm. Approximately  $5 \times 10^4$  cells per well (24 well plates) may be seeded one day before the transfection experiment. TfXTM-10 (liposome) transfection reagent (Promega) containing the cationic lipid component may be used according to the manufacturer's protocol. We will use charge ratios of 2:1 and 4:1 of liposome to nucleic acid for delivery of thioaptamers and this should be appropriate for delivery of RNA. A 400  $\mu$ l volume of nuclease-free water may be added to the vial of liposome reagent and vortexed for 10 seconds to suspend the lipid film. The vial may then be heated to 65°C in a water bath for 1 minute. After vortexing again, the vial may be stored at -20 C° overnight. TC-83 containing  $1 \times 10^4$  PFU/100  $\mu$ l will could be added to each well for 1 hour at 37°C, washed 2X with PBS, and then RNA aptamer or thioaptamer (a range of RNA concentrations will to be tested) may then be added to each well (3 replicates for each RNA thioaptamer or RNA aptamer concentration). The plates may be returned to the incubator for 1 hour and 1.25 ml of warm, complete medium may be added to each well. The culture medium may be sampled at 0, 8, 24 and 48 hours and the virus titer will be determined by standard plaque assay. For any aptamers that exhibit antiviral activity in vitro, a mouse model may be used to test for protection against lethal challenge with the virulent Trinidad donkey strain (parent of TC-83). The methods for in vivo delivery are being optimized in the arenavirus project.

[0081] An example of the application of the combinatorial library and selection methods to an assay allowing selection of a thio-siRNA is as follows: a split synthesis combinatorial chemistry method is to be developed to create a combinatorial library of  $[S_2]$ -ODN agents or mixed  $[O]/[S]/[S_2]$ -backbone ODN. A library can also be made in which the backbone is varied combinatorially but not the sequence and/or a library in which both backbone and sequence is varied combinatorially. Each unique member of the combinatorial library is to be attached to a separate support bead.

[0082] EXAMPLE 2 – Synthesis of a one bead-one monothioRNA library. Standard phosphoramidite (DNA and RNA) chemistry was used for the monothio-RNA library. A 0.5M  $^1$ H-tetrazole in acetonitrile was used as DNA activator. A 0.5M solution of DCI

(dicyanoimidazole) in acetonitrile was used as RNA activator. The libraries were prepared on a 1 umole scale of polystyrene beads (66-70 um). The 15-mer downstream and upstream primers, 5'-d(GGATCCGGTGGTCTG)-3' (SEQ ID NO.: 22) and 5'-d(CCTACTCGCGAATTC)-3' (SEQ ID NO.: 23) were synthesized in parallel on a two-column DNA synthesizer (Applied Biosystems Expedite 8909). Following the 5'-primer, the 31-mer sequences programmed on the synthesizer for the combinatorial monothio-RNA library.

[0083] The following RNA thioaptamers were synthesized and used for the following studies: 5'-r(GA\*UC\*CU\*GA\*AA\*CU\*GU\*UU\*UA\*AG\*GU\*UG\*GC\*CG\*AU\*C)-3' (SEQ ID NO.: 24) on column 1 and 5'-r(cU\*aG\*gA\*cU\*uG\*gC\*aC\*aA\*cC\*gU\*cA\*cA\*cU\*gC\*uA\*u)-3' (SEQ ID NO.: 25) on column 2 (where a lower case letter indicates a 3'-thioate linkage, an upper case letter indicates a 3'-phosphate linkage and an asterisk indicates a position at which a "split and pool" occurred in order to synthesize the combinatorial region of the monothio-RNA).

[0084] The coupling yield was typically upwards of 98.5% as determined by the dimethoxytrityl cation assay. Sulfurization chemistry used the Beaucage reagent. The fully protected monothio RNA combinatorial library with the non-cleavable linker beads were treated with 4 ml of a mixture of 3:1 (v/v) (28%) NH<sub>3</sub>:EtOH at 39°C for 21 hours. The beads were centrifuged, the supernatant was removed and the solid support was washed with double-distilled water. After lyophilization the solid support was treated with 2 ml of triethylamine hydrochloride (TEA-3HF) for 20 hours at room temperature. Again, the beads were centrifuged, the supernatant was removed and the solid support was washed with double-distilled water.

[0085] Several new approaches may be used to evaluate for assay/selection of thio-siRNAs. For example, consider a combinatorial library of thio-siRNAs targeting the GADPH gene, using an siRNA thioaptamer. Bead-bound combinatorial libraries (one bead-one thioaptamer) of the GADPH sequence siRNA are to be created using three alternate approaches:

[0086] (1) synthesize random monothioates in a split-pool chemically synthesized normal bead support and then put one or more beads into a well (of a 96 or higher multiple well-plate) and deprotect and release the deprotected RNA from the bead into the well. In some cases it may be useful to use a simple separation pack chromatographic purification of the ssRNA thioaptamer(s) in a well and then combining a group of several different combinatorial thioaptamers into another 96 well plate). One would then add just a normal phosphate complementary strand to each of the wells to convert to the ds siRNA thioaptamers. One would then add the liposome prep for cell

delivery and then the rest of the Ambion kit for rapid testing of inhibition of GADPH protein translation (using Ambion immunochemistry kit). Control may be, e.g., Ambion-supplied siRNA without any thiophosphate in the RNA backbone. It is also useful to confirm that the iodoethanol cleavage of the thioaptamer siRNA works as demonstrated for DNA thioaptamers, followed by  
5 gel electrophoresis or mass spectrometry (MS/MS) fragmentation to identify where the thio locations on the thioaptamer are in each well.

[0087] (2) Alternatively, a ddRNA (DNA derived siRNA) derived may be used from DNA templates. A non-combinatorial bead may then be used with ATP $\alpha$ S as an NTP cocktail to transcribe the siRNA in one well, another NTP[ $\alpha$ S] into another well. In one embodiment,  
10 varying ratios of ATP/ATP[ $\alpha$ S] may be used to add thiophosphate and some normal phosphate at different A, G, T or U sites on the sequence.

[0088] (3) Use of a non-cell based assay for RNAi activity of a siRNA (Kawasaki, et al., 2003). A non-cell based siRNA assay may use either or both monothioate and dithioate siRNA combinatorial beads by directly synthesizing our bead based monothioate libraries, placing one  
15 bead in each well, adding the complementary strand, and then without need of liposome for cell delivery, just assay following the Taira et al (Kawasaki, et al., 2003) non-cell based method. A longer tether from the bead to the siRNA thioaptamer may be needed in some circumstances, e.g., PEG or a long UUUUUU tether.

[0089] It must first be determined whether the physical attachment of the ds-siRNA molecule on  
20 the bead prevents the Dicer endonuclease of the RISC complex from binding to the mRNA and ds-siRNA thioaptamer (a long linker might be required to minimize such interference). If interference was significant, one would then design a means of releasing the ds-siRNA thioaptamer from the one bead in the well. The user then monitors the cleavage of the target mRNA by mass spectrometry, gels or ribozyme type assays.

[0090] EXAMPLE 3. Thioaptamer Gene Silencing. The following studies were conducted to demonstrate gene silencing using thioaptamers. Standard gene silencing studies were conducted with the following modification to the materials and methods. Phosphoromonothioate substituted siRNAs (thioaptamers) were used from the siSTARTER Luciferase Kit to conduct studies (Dharmacon, USA). The methods used were as described in the manufacturer's instructions. The  
25 term "luc" refers to the luciferase reporter gene. Briefly, the following siRNA duplexes were  
30 provided in the kit:

## Anti-luc siRNA-1

5'-GAU UAU GUC CGG UUA UGU AUU (SEQ ID NO.: 26)

UU CUA AUA CAG GCC AAU ACA U p-5'

## Anti-luc siRNA-2

5 5'-CUG AAU ACA AAU CAC AGA AUU (SEQ ID NO.: 27)

UU GAC UUA UGU UUA GUG UCU U p-5'

## Anti-luc siRNA-3

5'-UCC GGA AGC GAC CAA CGC C UU (SEQ ID NO.: 28)

UU AGG CCU UCG CUG GUU GCG G p-5'

10 Non-specific luc control siRNA

5'-AUG UAU UGG CCU GUA UUA G UU (SEQ ID NO.: 29)

UU UAC AUA ACC GGA CAU AAU C p-5'

[0091] The following siRNA thioaptamers duplexes were synthesized and used in these studies:

## Thioluc1(thio siRNA duplex 1)

15 5'- CU\*G A\*AU ACA AAU CA\*C A\*GA A UU -3' (sense) (SEQ ID NO.: 30)

3'-UU-GA C U UA UGU UUA GU G U CU UP-5'(antisense)

## ThioLuc2 (Thio siRNA duplex 2)

5'- C\*UG \*AAU ACA AAU C\*AC \*AGA A UU -3' (sense) (SEQ ID NO.: 31)

3'-UUGA C UUA UGU UUA GUG UCU UP-5'(antisense)

20 ThioLuc2 (Thio siRNA duplex 3)

5'- \*CU\*G AAU ACA AAU CAC \*AG\*A A UU -3'(sense) (SEQ ID NO.: 32)

3'-UU GA C UUA UGU UUA GUG UC U UP-5'(antisense)

## Control1 (control thio siRNA duplex 1)

5'- AU\*G U\*AU U GGCCU GU\*A U\*UA G UU -3' (sense) (SEQ ID NO.: 33)

25 3'-UUUA C A UA A CC GGA C A U A AU CP-5' (antisense)

\* = location of thio-modification.

- [0092] Base sequences from thioluc 1 through thioluc 3 are the same as anti-luc siRNA-2 and the sequence of control 1 is the same as non-specific luc control siRNA. The plasmid used was pGL3 plasmid (pGL3-expression vector) in a 1 x Universal buffer: 20 mM KCl, 6 mM HEPES-pH 7.5, 0.2 mM MgCl<sub>2</sub>. Transfection was accomplished using a TransIT-TKO transfection reagent: 2.5 µg/µl of non-liposomal polymer/lipid formulation. All RNAs were dissolved in RNase-free water. For detecting luciferase activity, the Promega Steady-Glo Luciferase Assay Buffer and Promega Steady-Glo Luciferase Assay Substrate kits were used according to the manufacturer's instructions. HeLa cells were grown in Opti-MEM (GIBCO) and when needed, supplemented with DMEM with L-glutamine, pyridoxine hydrochloride, high glucose and without sodium pyruvate. Additionally, we added the following to the medium (500 ml/ bottle) before using: 25 ml of 5% inactivated fetal bovine serum, 10.6 ml of 20 mM Hepes, 5 ml of 1.8 mM glutamine and 0.5 ml of 50.8 µM 2-mercaptoethanol. The HeLa Cells (human cervical epithelial adenocarcinoma, adherent; ccl-2) were obtained from ATCC, Rockville, MD. Other buffers used included 1x PBS buffer (GIBCO).
- [0093] Cells were plated in 96-well plate in triplicate for each condition using 200 µl of 1 X 10<sup>5</sup> HeLa cells/ml suspension. The cells were incubated for 24 hours at 37°C in 5% CO<sub>2</sub>. siRNA or thioaptamer siRNA duplexes and plasmid were resuspended in as follows: 200 µl siRNA were resuspended in Universal Buffer to each siRNA tube for a final concentration of 1.0 µM. Next, 408 µl of Universal Buffer were added to each thioRNA tube for a concentration of 100 µM. For the thioaptamers, 2 µl of 100 µM thio siRNA duplex solution were added to 198 µl of the Universal Buffer to each tube for a final concentration of 1 µM. Finally, for the reporter plasmid, 40 µl RNase-free water were added to the 10 µg of reporter plasmid for a final concentration of 250 ng/µl and 40 µl RNase-free water was added to the 10 µg of reporter plasmid tube for a final concentration of 250 ng/µl. The above were vortexed briefly and centrifuged.
- [0094] Formation of siRNA or thio siRNA-plasmid-lipid complex for transfection. Using all RNase-free solutions and tubes, the following mixtures were prepared in separate sterile polystyrene tubes:

Table 2A. TransIT-TKO/Plasmid dilution mixture (Mixture 1A)

Opti-MEM	186.0 µl	30
TransIT-TKO (2.5 µg/µl)	10.0 µl	
Reporter Plasmid (250 ng/µl)	4.0 µl	

Table 2B. TransIT-TKO dilution mixture (Mixture 1B)

Opti-MEM	37.2 ul
TransIT-TKO (2.5 ug/ul)	2.0 ul
Rnase-free Water	0.8 ul

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[0095] Next, the following mixtures were created using Tables 3A-F as a guide, to create Mixtures 3A, 3B, 3C, and 3D (siRNAs or thioRNAs), Mixture 3E (plasmid alone), and Mixture 3F (background control) in sterile 1.5-2.0 ml tubes.

Table 3A. siRNA duplex 1 dilution mixture (Mixture 2A)

Opti-MEM	29.7 ul
siRNA duplex 1 (1 uM)	3.3 ul
Mixture 1A	33.0 ul

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Table 3B. siRNA duplex 2 dilution mixture (Mixture 2B)

Opti-MEM	29.7 ul
siRNA duplex 2 (1 uM)	3.3 ul
Mixture 1A	33.0 ul

Table 3C. siRNA duplex 3 dilution mixture (Mixture 2C)

Opti-MEM	29.7 ul
siRNA duplex 3 (1 uM)	3.3 ul
Mixture 1A	33.0 ul

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Table 3D. Non-specific control duplex dilution mixture (Mixture 2D)

Opti-MEM	29.7 ul
Non-specific control (1 uM)	3.3 ul
Mixture 1A	33.0 ul

Table 3E. Plasmid alone dilution mixture (Mixture 2E)

Opti-MEM	29.7 ul
Rnase-free water	3.3 ul
Mixture 1A	33.0 ul

Table 3F. No Plasmid dilution mixture (Mixture 2F)

Opti-MEM	29.7 ul
Rnase-free water	3.3 ul
Mixture 1B	33.0 ul

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[0096] Each of these mixtures were mixed gently (not vortexed) and incubated at room temperature for 20 minutes. To these tubes, 264ul of DMEM were added to mixture 2A, 2B, 2C, 2D, 2E and 2F tubes respectively and again mixed gently (not vortexed).

[0097] Transfections were carried out as follows, carefully remove the medium from the cells to be transfected, carefully wash the cells 2 times with 50 ul of 1X PBS and add 100 ul mixture 2A ~ 2F to each well of 96-well plate (in triplicate for each condition) respectively and gently rocked

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the plate back and forth for even distribution of reagent. The cells were then incubated with transfection reagent mixture for 48 hours at 37°C in standard incubation conditions.

[0098] Luciferase Detection. Next, the entire contents of the Steady-Glo® Buffer were transferred to the bottle of Steady-Glo® Substrate at room temperature. The growth media from the cells was removed, being careful not to dislodge any of the cells. The cells were washed twice with 50 ul of 1 X PBS buffer, again taking care not to dislodge any of the cells and 100 ul of the Steady-Glo® mixture was added to each well and incubates at RT for 5 minutes. Finally, the luminescence in a luminometer is measured. Each of the samples and controls were measured and the means of the triplicates were calculated as follows:

$$\text{mean} = (\text{replicate 1} + \text{replicate 2} + \text{replicate 3})/3$$

[0099] The “No Plasmid” mean was subtracted from each sample (background subtraction) and divided by the background subtracted means by the “Plasmid Only” mean.

[0100] Phospho-siRNA silencing Studies. Figure 7 is a graph of normalized mean luciferase activity in HeLa cells. Luminescence units were normalized to plasmid alone controls, and which the following were used:

- 1 high silencer (siRNA duplex 1)
- 2 medium silencer (siRNA duplex 2)
- 3 low silencer (siRNA duplex 3)
- 4 control siRNA
- 5 plasmid alone

Raw data:

	High silencer	Medium silencer	Low silencer	Control siRNA	Plasmid only	blank
Replicate 1	1147	3647	8324	14564	12595	781
Replicate 2	1787	6519	11531	41383	33861	793
Replicate 3	1526	3519	10068	26587	14104	1247
mean	1487	4561.7	9974.3	27512	20188	940.33

Blank subtracted

	High silencer	Medium silencer	Low silencer	Control siRNA	Plasmid only
mean	546.67	3621.37	9033.97	26571.67	19247.67

% silencing to plasmid alone

	High silencer	Medium silencer	Low silencer	Control siRNA	Plasmid only
mean	2.84	18.81	46.94	163.54	100

[0101] Fig. 8 is a graph that shows normalized mean luciferase activity in HeLa cells. Luminescence units were normalized to plasmid alone controls, and which the following were used:

- 1 High silencer (siRNA duplex 1)
- 2 Medium silencer (siRNA duplex 2)
- 3 Low silencer (siRNA duplex 3)
- 4 Control siRNA
- 5 Plasmid alone

Raw data:

	High silencer	Medium silencer	Low silencer	Control siRNA	Plasmid only	blank
Replicate 1	1012	7231	13545	39057	1439	776
Replicate 2	2056	9817	38854	62856	66720	709
Replicate 3	839	4317	28603	897	26376	618
mean	1302.3	7121.7	27001	34270	31512	701

Blank subtracted

	High silencer	Medium silencer	Low silencer	Control siRNA	Plasmid only
mean	601.3	6420.7	26300	33569	30811

% silencing to plasmid alone

	High silencer	Medium silencer	Low silencer	Control siRNA	Plasmid only
mean	1.95	20.84	85.36	108.95	100

- 10 [0102] Figure 9 is a graph that shows silencing by thioaptamers, background subtracted and normalized mean luciferase activity in HeLa cells. Luciferase activity units were normalized to plasmid alone control, and which the following were used:

- 1 Thio RNA oligo duplex 1
- 2 Thio RNA oligo duplex 2
- 15 3 Thio RNA oligo duplex 3
- 4 Control thio RNA oligo duplex 4
- 5 Plasmid only

Raw data:

	thio RNA duplex 1	Thio-RNA duplex 2	Thio RNA duplex 3	control thio RNA duplex 4	Plasmid only	blank
Replicate 1	1679	1777	1298	1718	1352	786
Replicate 2	1574	1733	2062	4624	3718	931
Replicate 3	1205	1640	1299	5115	3932	1143
mean	1486.0	1716.7	1553.0	3819.0	3000.7	953.3

- 20 Blank subtracted

	thio RNA duplex 1	Thio-RNA duplex 2	Thio RNA duplex 3	control thio RNA duplex 4	Plasmid only
mean	532.7	763.4	599.7	2865.7	2047.4

% silencing to plasmid alone

	thio RNA duplex 1	Thio-RNA duplex 2	Thio RNA duplex 3	control thio RNA duplex 4	Plasmid only
mean	26.0	37.3	29.3	140.0	100

- [0103] Figure 10 is a graph that shows the effect of a thioaptamer on siRNA gene silencing in HeLa cells, and which the following were used:

- 1 phosphoro siRNA duplex 2  
 2 Thio-RNA oligo duplex 1  
 3 Thio-control RNA oligo duplex 4  
 4 Phosphoro control siRNA  
 5 plasmid alone

[0104] Figure 11 is a graph that shows the effect of a thioaptamer on siRNA gene silencing in HeLa cells, and which the following were used:

- 1 phosphoro siRNA duplex 2  
 2 Thio-RNA oligo duplex 1  
 10 3 Thio-control RNA oligo duplex 4  
 4 Phosphoro control siRNA  
 5 plasmid alone

Raw data:

	Phosphoro-siRNA duplex 2	Thio-RNA duplex 1	Thio-control RNA duplex	Phosphoro-control siRNA duplex	Plasmid only	blank
Replicate 1	965	912	4420	1545	4097	460
Replicate 2	1103	2407	18855	5028	13274	546
Replicate 3	918	1706	18147	11037	13637	610
mean	995.33	1675	13807	5870	10336	538.67

15 Blank subtracted

	Phosphoro-siRNA duplex 2	Thio-RNA duplex 1	Thio-control RNA duplex	Phosphoro-control siRNA duplex	Plasmid only
mean	416.66	1136.33	13268.33	5331.33	9797.33

% silencing to plasmid alone

	Phosphoro-siRNA duplex 2	Thio-RNA duplex 1	Thio-control RNA duplex	Phosphoro-control siRNA duplex	Plasmid only
mean	4.25	11.60	135.4	54.42	100

[0105] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0106] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it

will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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